

Cardiovascular Imaging

Spontaneous Echo Contrast Videodensity Is Flow-Related and Is Dependent on the Relative Concentrations of Fibrinogen and Red Blood Cells

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OBJECTIVES	The purposes of the present study were to: 1) determine whether fibrinogen (Fg) is the plasma protein responsible for spontaneous echo contrast (SEC), and 2) investigate modulators of SEC.
BACKGROUND	Spontaneous echo contrast has been linked to the development of thromboemboli. The blood products and their interaction responsible for SEC formation have not been fully elucidated.
METHODS	Blood echogenicity was examined with the use of quantitative videodensitometry over a controlled range of flow velocities in an in vitro model. Human blood samples were analyzed in a manner to methodically eliminate individual blood components from whole blood to determine which components are responsible for the formation of SEC.
RESULTS	The videodensity (VD) of whole blood was found to be flow-dependent, with higher VD at lower flow rates, and correlated with visually dense SEC. The following blood products produced faint VD values: washed red blood cells (wRBCs), platelet-depleted plasma, Fg, defibrinated plasma, wRBCs plus defibrinated plasma, and physiologic saline. The VD of wRBCs increased incrementally as increasing concentrations of Fg were added. At each hematocrit (Hct) range, as Fg concentration increased, the SEC became denser, and the VD level also increased until a plateau level was reached that was distinct for each Hct. The addition of sialic acid, which inhibits RBC-RBC aggregation, decreased the amount of SEC, even in the presence of Fg.
CONCLUSIONS	These results demonstrated that Fg-mediated RBC aggregation may be responsible for SEC generation. Furthermore, a unique stoichiometric relationship exists between Fg and RBC concentrations that is necessary for blood echogenicity. (J Am Coll Cardiol 2003;41:603–10) © 2003 by the American College of Cardiology Foundation

Spontaneous echo contrast (SEC), or “smoke,” is a swirling gray haze of variable density detected by transthoracic or transesophageal ultrasound imaging of the chambers of the heart and great vessels under conditions of low flow (1). Spontaneous echo contrast has been linked to the development of thromboemboli in low-flow clinical states such as mitral stenosis, atrial fibrillation, cardiomyopathy, and left ventricular aneurysm (2–5). However, whether SEC itself contributes directly to thrombus formation or is just a marker of a prothrombotic milieu is unclear.

Numerous investigators have sought a better understanding of the mechanism responsible for SEC formation (6–12). Our previous study documented that SEC is dependent on red blood cell (RBC) and plasma protein interactions (9). Several reports have postulated that fibrinogen (Fg), given its major role in RBC aggregation, would be a likely contributor to SEC formation (12–18). However, these studies were performed in vitro at ambient temperature, therefore providing no direct evidence of this sug-

gested effect at body temperature. Thus, we sought to determine the components of SEC under diverse conditions, including human body temperature.

Therefore, the purposes of the present study were to: 1) determine whether Fg is the plasma protein responsible for SEC, and 2) investigate the modulators of SEC density.

METHODS

Subjects. Ten healthy human male and female volunteers (age range, 25 to 45 years) served as donors of blood products, after providing written informed consent for an institutional review board approved study protocol. Each volunteer may have donated blood on more than one occasion. Blood samples were all anticoagulated with heparin (5 U/ml). For each experiment multiple study samples were prepared from single blood donations (90 ml) from each volunteer. A total of 90 blood samples were examined. The study was approved by the institutional review board.

In vitro models. Blood samples were analyzed in two different in vitro models: 1) a large cylindric continuous flow chamber (30-mm diameter) in which blood was circulated

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Abbreviations and Acronyms

Fg	=	fibrinogen
Hct	=	hematocrit
plt	=	platelet
RBC	=	red blood cell
SEC	=	spontaneous echo contrast
VD	=	videodensity
WBC	=	white blood cell
wRBC	=	washed red blood cell

at varying shear rates from a small tube (3.8-mm diameter) (Figs. 1A and 2), in a 3-ml mixing chamber (Fig. 1B)

CONTINUOUS FLOW CHAMBER. Blood (90 ml) was circulated by a peristaltic pump through a narrow (3.8-mm diameter) plastic tube into a larger cylindric expansion chamber (30-mm diameter) at different velocities at body temperature (37°C). A linear 7.5-MHz phased array, 128-element transducer (Acuson, Mountain View, California) was applied longitudinally to the chamber and fixed in position (Fig. 1A). The video images were recorded on a one-half inch VHS recorder and analyzed by two independent observers. Videodensity (VD) was assigned using a commercially available video-densitometry analyzer (Nova Microsonics, New Jersey) based on a relative scale of 0 to 100 (0 = black, 100 = white). Videodensity was analyzed in three 1-cm² regions of interest in the inlet of the larger

chamber: 1 cm distal to inlet port at the center, and 1 cm both superior and inferior to the central inflow jet. Videodensity was measured at different velocity rates (0 to 150 ml/min) in the center of the flow and in the periphery of the chamber at the three fixed locations.

MIXING CHAMBER. To evaluate the echodensity of a larger number of blood product combinations at both stasis and under shear forces, we designed an apparatus for smaller blood volumes that would facilitate temperature control (Fig. 1B). This design required a sample of only 2.5 ml of blood, which was placed into a 3-ml cylindrical syringe containing a small teflon-coated stirring magnet (3 × 6 mm²) at its bottom. The blood was applied via a side-arm device, and the syringe was then sealed with a plastic cap. To obtain echo images free of reverberations, the syringe was placed horizontally in a 60-ml water bath at room and body temperature, and fixed in position over a magnetic stirrer adjusted to stir the syringe at varying rates of agitation. The echo transducer was applied longitudinally to the chamber and fixed in position with a C-arm device. Two independent observers using the same video densitometry analyzer used for the larger flow chamber analyzed the recorded video images. The region of interest analyzed was a 1-cm² cross-section obtained 1 cm above the agitation produced by the magnetic stirrer. Imaging was performed at varying rates of magnetic stirring and then continuously for 6 min after the stirring was stopped.

Echocardiographic standardizations. The same echo ultrasound power transmission was used for all imaging. After testing multiple combinations of machine settings, the following were considered to be the optimal for imaging of SEC in both the flow and mixing chambers: 1) gray scale, 2) log compression = ±40 dB, and 3) gain = +20 dB.

To calibrate the two measuring systems (the flow chamber and mixing chamber), video densitometry measurements for the normal heparinized whole blood (hematocrit [Hct] = 44% and Fg = 320 mg/dl) were obtained at stasis. The VD value of images obtained at stasis from the flow chamber (VD = 69.2) was less than the value obtained by imaging the mixing chamber in the water bath (VD = 78.0). Spontaneous echo contrast was subjectively defined as visible swirling haze or smoke-like echo signals and was measured objectively by VD at stasis.

Study of blood components. The effect of blood components on SEC formation was studied by using a systematic deletion of components from whole blood. This allowed us to observe the interactions of different components of blood and, thereby, determine which promote SEC formation. We first utilized the continuous, large volume flow chamber to analyze which blood products alone or in combination produced SEC. Subsequently, we verified these results using the small mixing chamber, enabling evaluation of a large quantity of samples using a smaller, more manageable volume of blood. Shown in Table 1 are the blood components and the sequence in which they were analyzed.

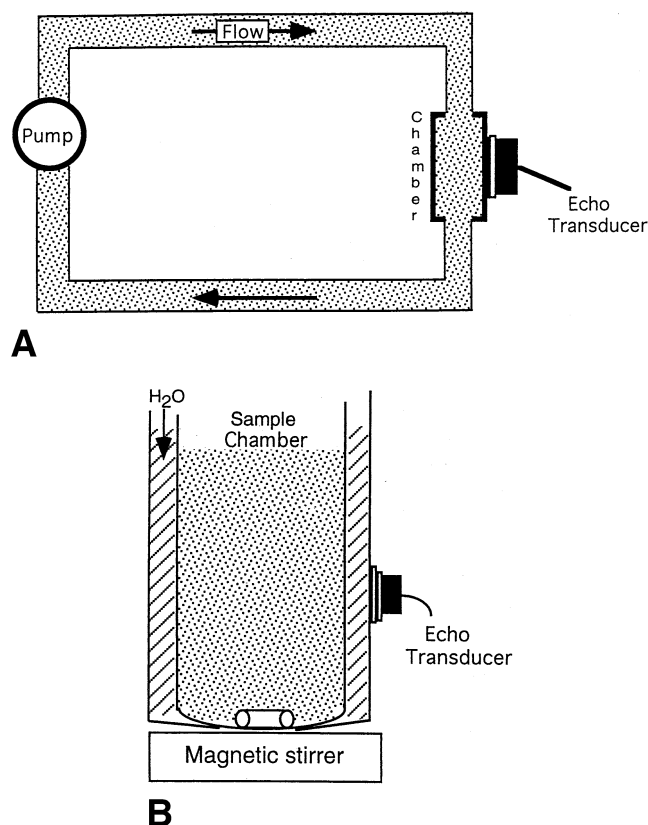


Figure 1. Pictorial representation of the flow chamber (A) and mixing chamber (B).

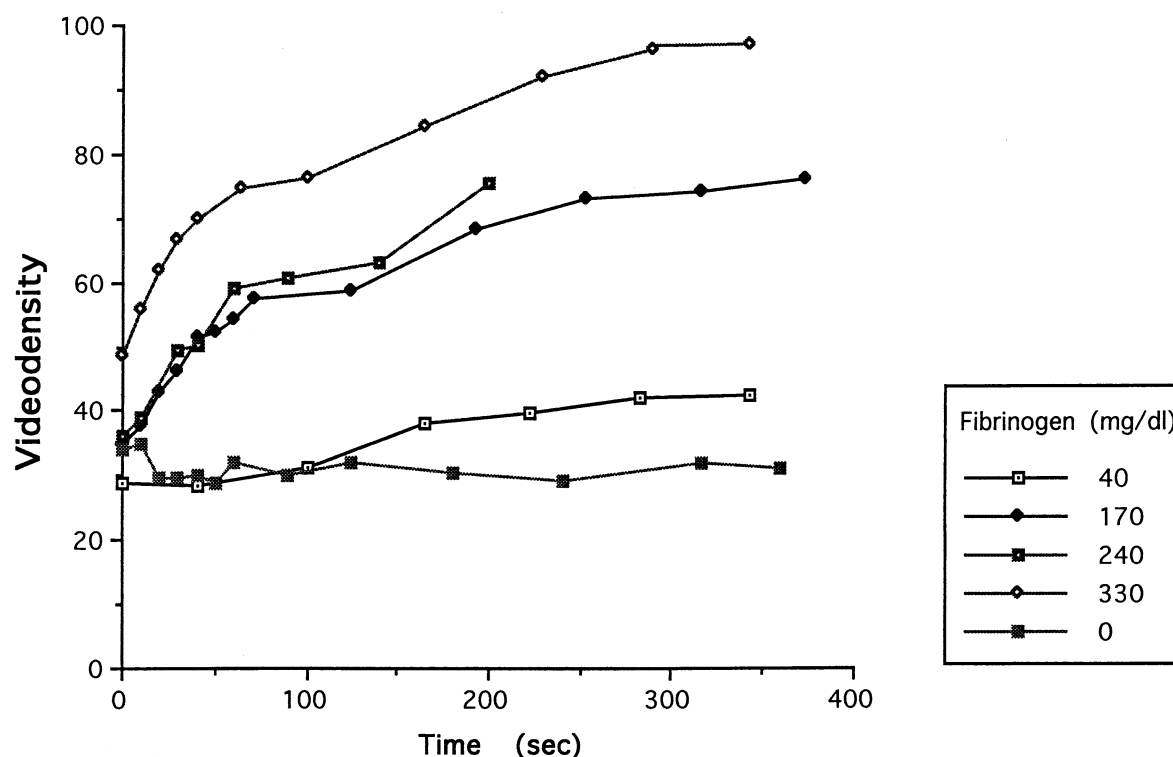


Figure 2. Spontaneous echo contrast videodensity over time using blood (hematocrit = 44%) with varying fibrinogen levels.

Whole blood, defined as RBCs, white blood cells (WBCs), plasma Fg, and platelets (plt) were utilized initially. The concentrations of the whole blood components were as follows: Hct = 42% to 46%, WBCs = $6.9 \times 10^3/\mu\text{l}$, plts = 200 to $350 \times 10^3/\mu\text{l}$, and plasma Fg levels = 175 to 300 mg/dl.

Washed red blood cells (wRBC) were obtained by centrifuging whole blood at 1,800 *g* at room temperature for 15 min. The plasma and buffy coat were discarded, and the RBC were resuspended in physiological saline to obtain the desired Hct ranges. Because of difficulty in reproducing exact Hct concentrations, four ranges were used: very low (7 to 14), low (27 to 35), medium (39 to 47), and high (53 to 65).

Platelet poor plasma was obtained by centrifuging whole blood at 800 *g* at room temperature for 10 min. The supernatant plt-rich plasma and the buffy coat were transferred to another tube and recentrifuged at 1,800 *g* at room

temperature for 10 min to obtain a plt-poor plasma supernatant (plt = 20 to $40 \times 10^3/\mu\text{l}$).

Defibrinated blood was obtained by centrifuging whole blood at 1,800 *g* for 10 min at room temperature, and the plasma was separated. Defibrinating snake venom enzyme, Ancrod (Sigma, St. Louis, Missouri: Agkistrodon rhodostoma venom, Malayan Pit Viper) was added to a final concentration of 1 U/dl. The Ancrod and plasma were first incubated at 37°C for 20 min and then placed at -20°C for 10 min to slow down the reaction rate. The mixture was then centrifuged at 1,800 *g* for 5 min at room temperature. The supernatant and defibrinated plasma had no detectable clottable Fg (Diagnostica Stago St 4: American Bioproducts Company, Parsippany, New Jersey). The defibrinated plasma had no detectable, residual ancrod activity as judged by its inability to defibrinate a sample of Fg (40 mg/dl before incubation and 38 mg/dl after incubation).

Lyophilized human Fg (Sigma, 90% clottable) was dis-

Table 1. Contents of Various Blood Components

Components	RBC	Fibrinogen	WBC	Platelets	Serum
Whole blood	+	+	+	+	+
Washed RBCs	+	-	-	-	-
Platelet-poor plasma	-	+	-	-	+
Reconstituted blood	+	+	-	-	+
Fibrinogen	-	+	-	-	-
Defibrinated plasma	-	-	-	-	+
Defibrinated blood	+	-	+	+	+
Normal saline	-	-	-	-	-

RBC = red blood cell; WBC = white blood cell.

solved in deionized water to obtain the desired concentrations (38, 60, 170, 240, and 331 mg/dl).

Reconstituted blood was obtained by adding Fg (in increments to achieve concentrations of 38, 60, 170, 240, and 331 mg/dl) to a mixture of defibrinated plasma and wRBCs.

Lastly, defibrinated “blood” was obtained by adding wRBCs to defibrinated plasma.

Normal saline (0.9%) served as our protein-free control.

These blood products (anticoagulated with heparin 5 U/ml) and control solution were all evaluated in the continuous flow chamber at differing flow rates (stasis, 25 ml/min, 50 ml/min, 60 ml/min, 75 ml/min, and 125 ml/min).

Given that SEC was produced only when combining RBCs and Fg in the continuous chamber, we were able to focus on the RBC-Fg interaction in the mixing chamber. Fibrinogen and plts alone served as controls for this part of the study. To determine the dependence of SEC on RBC-RBC interaction, sialic acid, an agent that opposes RBC interaction by contributing to the electrorepulsive forces on the RBC surface, was added to the reconstituted mixture of washed red cells (Hct = 42%) + Fg (493 mg/dl). Sialic acid (Sigma) was prepared to final concentrations of 4 mM and 40 mM by dissolving it in normal saline.

Each of the above blood samples was analyzed for the following: Hct, WBC, and plt counts, and Fg concentration, both before and after echo imaging.

Photomicrographs of SEC. To evaluate the role of RBC aggregates in SEC formation, 100 μ l samples of wRBCs + Fg were slowly taken from the syringe 6 min after stasis had been established and placed on a glass microscope slide. The slides were viewed using 10 \times oculars and a 40 \times phase contrast objective (Nikon, Melville, New York) by two independent observers blinded to the Hct and Fg levels. Each observer scored the RBC aggregation as “none,” “low,” or “high,” and photomicrographs were taken with a (Nikon) camera.

The interobserver correlation for VD measurements for 63 separate video images assessed by two reviewers was excellent ($r = 0.99$).

RESULTS

The large flow chamber was utilized as an attempt to reproduce in vivo interactions between various blood components. Saline served as our cell- and protein-free control, and produced a VD value of zero at all flow rates. We then evaluated the blood components systematically—individually and in combination—as described in the Methods section. As shown in Table 2, the VD is directly related to an interaction between RBCs and Fg. For example, whole blood, which contains all blood components, has high VD values compared with isolated Fg, RBCs, plt-poor plasma, or defibrinated plasma. Furthermore, its VD is higher at stasis (VD = 69) compared with

Table 2. Videodensity Values of Various Blood Products

Blood Product	Videodensity*	
	Flow Chamber	Mixing Chamber
Physiologic saline	0	0
Fg (700 mg/dl)	9.4	9.2
wRBC	30.0	34.0
Platelet poor plasma	35.0	
Whole blood	69.0	78.0
Defib plasma	31.0	
Defib plasma + wRBC	31.0	
Defib plasma + wRBC + Fg (330 mg/dl)	96.0	
wRBC (Hct = 42) + Fg (493 mg/dl)		86.0
wRBC (Hct = 42) + Fg (490 mg/dl + sialic acid) (4 mmol) (40 mmol)		64.0
		34.0

*Studies conducted at stasis.

Defib = defibrinated; Fg = fibrinogen; Hct = hematocrit; wRBC = washed red blood cells.

flow rates of 125 ml/min (VD = 12.9). In contrast, the VD of lone Fg is equal to 9.4, and that of wRBCs, plt-poor plasma, and defibrinated plasma is 30, 35, and 31, respectively.

To test the dependence of VD level on Fg, Fg was added to wRBCs and defibrinated plasma. As Fg was incrementally added to the wRBC/defibrinated plasma mixture, the VD increased from faint (in the absence of Fg) to a VD of 96 when the Fg concentration reached 330 mg/dl. As with whole blood, the VD diminished with increasing shear rates (characterized by higher velocities). The VD of reconstituted blood was also evaluated as a function of time. As previously stated, the VD decreases with increasing flow. When flow is abruptly terminated (time = 0), SEC appeared throughout the chamber. As time elapsed, SEC became denser and eventually reached a plateau (Fig. 2). It appears that the higher the Fg concentration, the longer it took for the VD to reach the plateau.

Importantly, these results were reproducible in the mixing chamber, thereby enabling us to readily test a large number of samples in a more manageable in vitro system. As in the larger continuous chamber, Fg solution was echo-free at stasis and at all flow rates with a VD = 9.2 (Table 2). The interdependency of Hct and Fg levels was substantiated by analyzing a larger number of blood samples with different Hct and Fg concentrations. The results are as follows:

1. At the very low Hct range (7% to 14%), no SEC was detected for Fg concentrations below 300 mg/dl. As the Fg concentration increased to 1,800 mg/dl, the VD gradually increased, reaching a plateau at Fg concentrations >2,000 mg/dl (Fig. 3A).
2. At the low Hct group (27% to 35%), VD increased rapidly as Fg concentration was increased from 100 to 200 mg/dl. A VD plateau was reached at 300 to 400 mg/dl of Fg concentration (Fig. 3B).

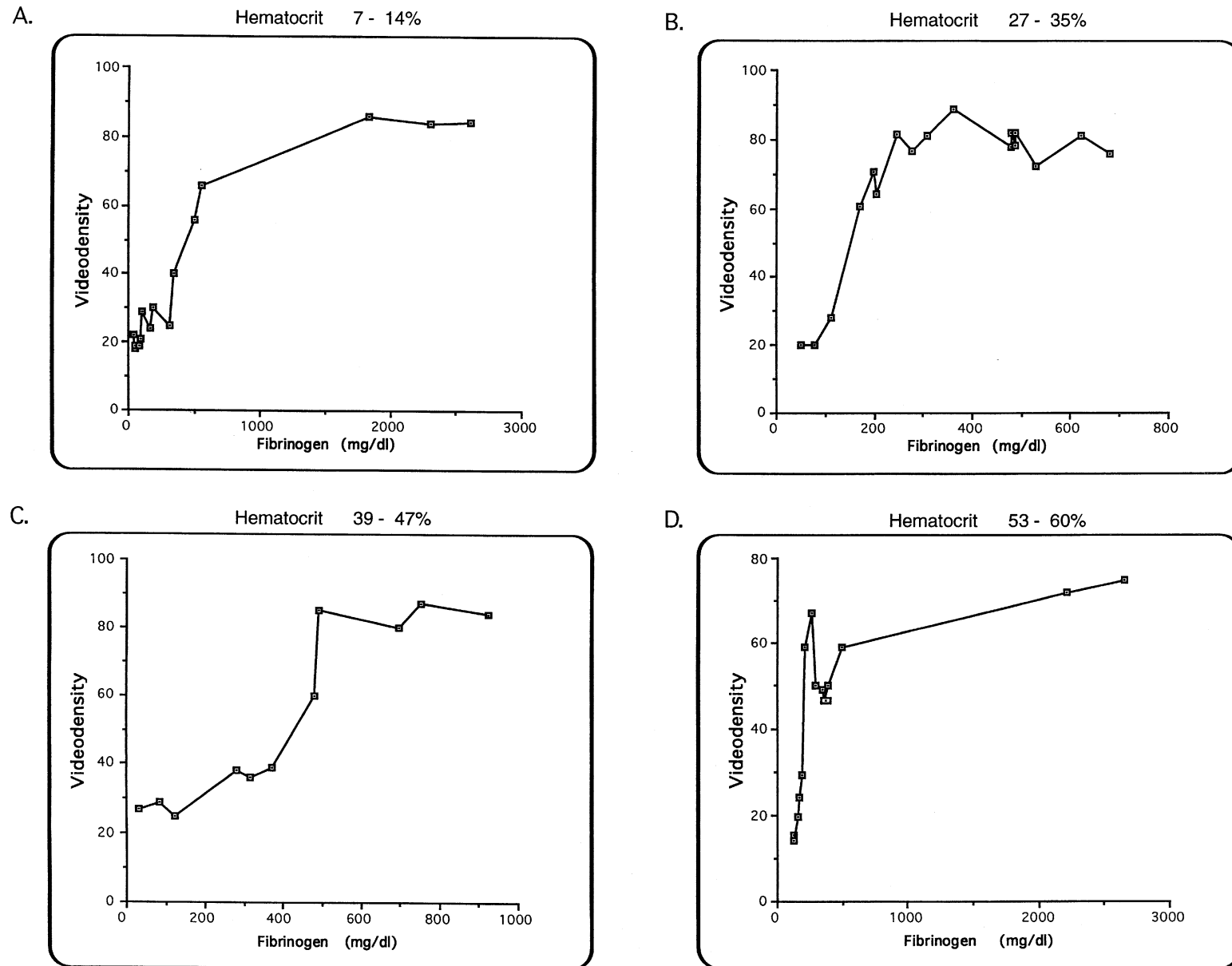


Figure 3. Blood spontaneous echo contrast videodensity as a function of increasing fibrinogen levels for different hematocrit concentrations. (A) Videodensity value versus fibrinogen concentration for the hematocrit range 7% to 14%. (B) Videodensity value versus fibrinogen concentration for the hematocrit range 27% to 35%. (C) Videodensity value versus fibrinogen concentration for the hematocrit range 35% to 47%. (D) Videodensity value versus fibrinogen concentration for the hematocrit range 53% to 60%.

3. At the physiologic range of Hct (39% to 47%), the major increase in VD occurred at Fg concentration between 400 to 500 mg/dl (Fig. 3C).
4. For the high Hct group (53% to 65%), SEC was achieved at Fg level of 250 mg/dl, and reached a VD plateau at 600 mg/dl (Fig. 3D).

By analyzing the VD by concentration of Fg graph for each set of Hct concentrations, we noted a specific stoichiometric relationship between Fg concentration and RBC interaction to generate SEC.

The addition of plts ($580 \times 10^3/\mu\text{l}$) to a mixture of wRBCs (Hct = 27%) and Fg (621 mg/dl) increased VD from 65 to 85 at stasis.

Lastly, the addition of sialic acid to wRBCs and Fg was analyzed. The VD values of the wRBCs and Fg (Hct = 42% and Fg = 490 mg/dl yielded a VD = 86) decreased as sialic acid was added (sialic acid concentration of 4 mM yielded a VD = 64; sialic acid concentration of 40 mM yielded a VD = 43). The decrease in VD suggested that sialic acid may prevent SEC formation by increasing the negative electrorepulsive charges.

The use of microscopic evaluation further supported the findings of RBC-Fg interdependence on SEC formation. The photomicrographs (Figs. 4A to 4C) demonstrated that RBCs alone formed minimal aggregates with a VD value of 24.0 (Fig. 4A). However, RBC aggregation (rouleaux formation) and VD increased as the Fg concentration increased (Fg = 349 to 493 mg/dl), in concert with an increase in the VD value (VD = 50 and 59, respectively) (Figs. 4B and 4C).

DISCUSSION

Previous work from our group had established that RBCs and plasma protein components, and not plts, were responsible for SEC formation. We carried out this study to investigate the plasma component responsible for SEC generation. A previous *in vitro* study done at ambient temperature by Fatkin et al. (12) demonstrated that a protein-mediated red cell aggregation is the mechanism of SEC in human blood, and suggested that red cells and Fg were required for blood echogenicity. Our study, which quantified SEC using *in vitro* VD measurements of human blood at body temperature, confirms that SEC is dependent on the interaction between RBCs and Fg, and their flow rates. We have also demonstrated that Fg is the principal plasma protein determinant of SEC generation by eliminating the echogenicity of whole blood by defibrination and generating SEC by adding Fg to wRBCs. In contrast to Fatkin et al. (8), we demonstrated the complex interaction between RBCs and Fg by titrating increasing Fg concentrations to fixed RBC concentration, as well as titrating increasing RBC concentrations to fixed Fg levels. Furthermore, we have found a stoichiometric relationship between Fg and red cells in SEC formation, such that, at lower Hct ranges, a higher concentration of Fg is required before SEC

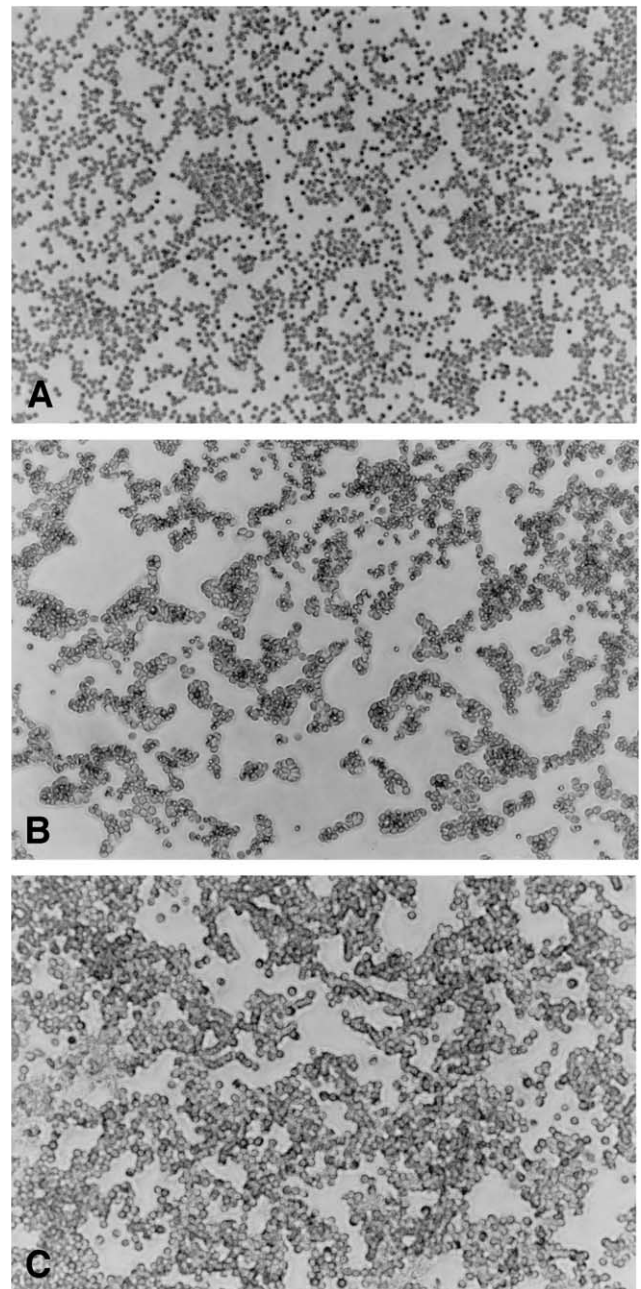


Figure 4. (A) Photomicrograph of red blood cell aggregates: washed red blood cells (hematocrit = 58%) and fibrinogen = 166 mg/dl. The videodensity was 24.0. (B) Photomicrograph of red blood cell aggregates: washed red blood cells (hematocrit = 58%) and fibrinogen 349 = mg/dl. The videodensity was 50.0. (C) Photomicrograph of red blood cell aggregates: washed red blood cells (hematocrit = 58%) and fibrinogen = 493 mg/dl. The videodensity was 59.0.

is observed, while the reverse is true at higher Hct ranges. This suggests an important relationship between the number of RBCs and Fg molecules. Lastly, we demonstrated that, with the addition of sialic acid, an agent that opposes RBC aggregation, SEC is inhibited, strengthening the argument that greater red cell “clumping” leads to greater levels of SEC.

Importance of SEC. Spontaneous echo contrast has been implicated as a marker of an inflammatory state preceding blood clot formation and subsequent embolic events. It is utilized as a determinant of risk when evaluating patients with such conditions as atrial fibrillation, dilated left atria in mitral stenosis, or in dilated cardiomyopathic ventricles or large apical aneurysms (18-23). Independent studies have demonstrated that stroke or other embolic event rates are higher in patients with SEC compared with patients without SEC (19,24). Furthermore, patients with SEC also have significantly reduced survival ($p = 0.025$) compared with those without SEC (19). Prospective studies evaluating the changes in SEC fail to demonstrate the resolution of SEC in the presence of antithrombotic therapy (20). This raises the question of the determinants of SEC as well as potential treatments, given SECs strong association with adverse thromboembolic events.

Determinants of SEC. Several clinical studies have suggested a variety of etiologies involved in the generation of SEC. Blood factors such as Fg, RBCs, plts, and leukocytes, as well as the interaction between these components, have all been implicated in SEC formation (6,7).

In addition, structural properties of the heart and great vessels have been correlated with SEC formation. For example, dilated left atria, cardiomyopathic ventricles, and dilated aortic segments are all independently associated with SEC formation (18,21-23,25). Furthermore, SEC has also been correlated to the flow velocity of blood (26,27). Regions characterized by low-flow, which correspond to low shear rates, have been correlated with increased formation of SEC. This is especially true of blood flow patterns in the left atrial appendage (3,28). In the present study, the RBC-Fg interaction was responsible for the generation of SEC, and that the quantity of SEC (as measured by VD) varied by RBC and Fg concentration, as well as on the velocity of their flow.

Quantification of SEC. We utilized videodensitometry to objectively grade the severity and to quantify the amount of SEC. We had a significant interobserver agreement for quantifying SEC videodensitometry. Videointensity has been previously validated as a reliable and rapid tool to quantify SEC (29). Recent studies have reported on other techniques to quantify SEC (30,31).

Relationship of SEC to thrombus formation. Though multiple groups have attempted to characterize the determinants of SEC, the relationship between SEC to actual thrombus formation has not been fully elucidated (5). Though SEC is plt-independent, plts contribute to thrombus, and may be necessary for clinical clot formation. Clinically, SEC can be imaged in cardiac chambers of patients even if they are anticoagulated with heparin or warfarin. Blood used in our preparations were anticoagulated, which prevented clotting but did not interfere with SEC formation. It is notable, however, that Fg not only promotes SEC, but is also able to support plt aggregation by binding to the plt glycoprotein IIb/IIIa receptor complex

(32). It has also been shown that plt membrane activation markers are expressed in the embolic and pre-embolic states of patients with nonrheumatic atrial fibrillation. A more recent study, which examined blood from patients with and without SEC, found a higher activation and aggregation of plts and plt-leukocyte aggregates in venous than arterial blood, as well as in the left atrial chamber compared with the right atrial chamber in patients with mitral stenosis (33). This implies a higher level of plt activity of blood in the venous system or in generally low-flow states and may support a role for plt aggregates in the pathogenesis of SEC (34). Our study, however, has demonstrated that plts are not necessary nor are they directly related to SEC formation, which is substantiated by prior studies (5). Interestingly, our study demonstrated that Fg concentrations in the range of 400 to 500 mg/dl were associated with the greatest SEC generation in the Hct range from 39% to 47%. Those clinical studies demonstrated similar levels of Fg in patients presenting with acute coronary syndromes (35,36).

Study limitations. We have demonstrated that SEC formation is dependent on RBC interactions with Fg. However, the VD value of whole blood with the same Hct and Fg concentration (175 to 350 mg/dl) is higher than what was obtained by reconstituting wRBCs with Fg. The most likely reason for this is that other plasma properties also contribute to SEC formation.

Differences in VD between the flow chamber and the small mixing chamber may have been due to several factors. The larger flow chamber utilized 90 ml of blood compared with only 3 ml in the small syringe. Thus, ultrasound attenuation due to larger volume and distance to sample volume may have affected the VD measurements, with higher values for similar solutions measured in the smaller chamber. The smaller chamber may have facilitated more uniform mixing compared with the larger chamber in which a narrow jet entering a large static volume may generate eddy currents of flow.

Conclusions. In summary, we have demonstrated that Fg can affect RBC rouleaux formation in a flow-dependent, plt-independent phenomenon and is detectable by ultrasound as SEC. The observation that Fg concentration required to achieve maximal VD varied with the Hct concentration suggested an important relationship between RBCs and Fg in producing SEC. Future treatments aimed at preventing protein-RBC interactions as demonstrated by direct RBC-Fg interactions in our study, or even RBC-RBC interactions, may provide alternatives to anticoagulant and antiplatelet therapy to reduce cardiac thromboembolic risk.

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